Diabetogenic Effects of Glucocorticoid Drugs: The Knowns and The Unknowns

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Chapter 12

Angiopoietin-Like Protein 4 is Differentially Regulated by Glucocorticoids and Insulin in *vitro* and in *vivo* in Healthy Humans

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ABSTRACT

Objective: Angiopoietin-like protein 4 (Angptl4) is a circulating inhibitor of plasma triglyceride clearance via inhibition of lipoprotein lipase. The aim of the present study was to examine the regulation of Angptl4 by glucocorticoids and insulin *in vivo* in humans, since these factors regulate Angptl4 expression *in vitro*.

Research Design and Methods: In a randomized, placebo-controlled, double-blind, dose-response intervention study, 32 healthy males (age: 22±3 years; BMI 22.4±1.7 kg m^{-2}) were allocated to prednisolone 30 mg once daily (n=12), prednisolone 7.5 mg once daily (n=12), or placebo (n=8) for two weeks. Angptl4 levels and lipid metabolism were measured before and at 2 weeks of treatment, in the fasted state and during a two-step hyperinsulinemic clamp. Additionally, human hepatoma cells were treated with dexamethasone and/or insulin.

Results: Compared to placebo, prednisolone treatment tended to lower fasting Angptl4 levels (P=0.073), raised fasting insulin levels (P=0.0004) and decreased fasting nonesterified fatty acid concentrations (NEFA) (P=0.017). Insulin infusion reduced Angptl4 levels by 6% (plasma insulin~200 pmol/l, P=0.006) and 22% (plasma insulin~600 pmol/l, P<0.0001), which was attenuated by prednisolone treatment (P=0.03). Prednisolone 7.5 mg and 30 mg dose-dependently decreased insulin-mediated suppression of lipolysis (by 11±5% and 34±6% respectively). Prednisolone 30 mg enhanced fasting triglyceride levels (P=0.028). Plasma Angptl4 was not related to prednisolone-induced changes in lipid metabolism. In human hepatoma cells, dexamethasone increased Angptl4 mRNA expression and protein secretion, whereas insulin had the opposite effect.

Conclusions: Insulin lowers plasma Angptl4 levels in humans by lowering NEFA and by inhibiting Angptl4 expression and release. Glucocorticoids counteract insulin-mediated suppression of Angptl4.

Clinical Trial Registration: ISRCTN83991850

The regulation of circulating lipids is tightly controlled. An important endocrine factor involved in the regulation of circulating lipid levels is angiopoietin-like protein (Angptl) 4. Angptl4 is a 50kD protein secreted by a variety of tissues including liver, white adipose tissue and skeletal muscle [1-3]. Studies in various animal models have shown that Angptl4 increases plasma triglyceride levels [4-6], by inhibiting plasma lipoprotein lipase (LPL) activity [7]. Accordingly, carriers of a rare dysfunctional variant of the Angptl4 gene exhibit low plasma TG levels [8]. Besides raising plasma TG, Angptl4 increases plasma non-esterified fatty acids (NEFA) by stimulating white adipose tissue lipolysis [6, 9].

Expression of Angptl4 is stimulated by fatty acids in several mouse tissues via activation of the peroxisome-proliferator activated receptors α, γ and δ [3, 10-12]. Consistent with a role of PPARs in regulating Angptl4 production in humans, synthetic agonists of PPARα and PPARγ raise plasma Angptl4 levels in human subjects [3, 4, 13].

Insulin, on the other hand, may suppress Angptl4 levels. In 3T3-L1 adipocytes, insulin treatment reduced Angptl4 expression, an effect that was attenuated by pharmacological inhibition of the insulin-signaling cascade and by tumor necrosis factor-α treatment [14]. Recently, a possible role for glucocorticoids (GCs) in the regulation of Angptl4 expression was suggested. Dexamethasone treatment was found to increase Angptl4 mRNA levels in primary hepatocytes and adipocytes, and in mouse liver and white adipose tissue. A glucocorticoid response element (GRE) was identified in the 3'-untranslated region of the Angptl4 gene, suggesting that Angptl4 represents a direct GC target gene [15]. Interestingly, Angptl4$^{-/-}$ mice were less susceptible to hypertriglyceridemia and hepatic steatosis following dexamethasone treatment [15]. Whether GCs are important regulators of Angptl4 levels in humans is presently unclear.

More recently, we conducted a study in which we observed that short term treatment with both low- and high-dose prednisolone in healthy men affected glucose metabolism and lipolysis, resulting in impaired insulin-stimulated suppression of endogenous glucose production and whole-body lipolysis [16]. Given the recent discovery of the involvement of Angptl4 in glucocorticoid-induced metabolic deregulation, we additionally determined Angptl4 plasma concentrations in these individuals before and after prednisolone treatment in samples that were collected during the study. We hypothesized that prednisolone would increase Angptl4 plasma concentrations and that changes in Angptl4 concentrations would be related to the observed prednisolone-induced alterations in lipid metabolism. Our experimental design also permitted to assess the effect of hyperinsulinemia on plasma Angptl4 concentrations. We hypothesized that insulin infusion would decrease Angptl4 concentrations and that

prednisolone would attenuate this effect by inducing insulin resistance. The study was supported by *in vitro* experiments using human hepatoma cells.

RESEARCH DESIGN AND METHODS

Clinical study

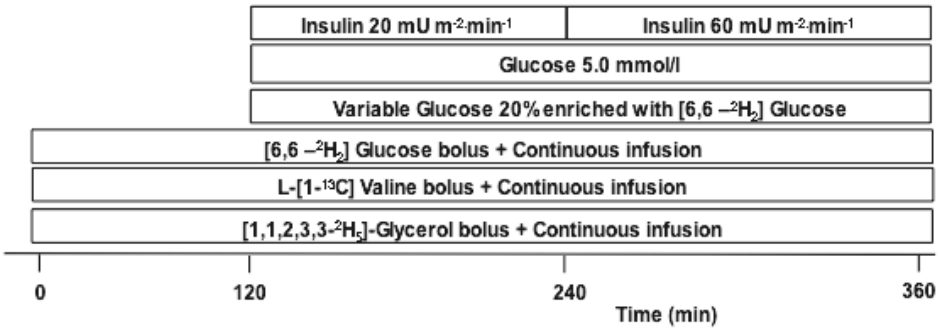
Participants: Thirty-two healthy normoglycemic males were included, following a screening visit during which history was taken and a physical examination, screening blood tests and an oral glucose tolerance test were performed. Inclusion criteria were: age between 18-35 years and body mass index (BMI) ≤ 25 kg/m^2. Exclusion criteria were any previous or current illness, use of any medication, first-degree relative with type 2 diabetes, smoking, shift work, a history of glucocorticoid use, excessive sport activities (i.e. more often than two times/week) and recent changes in weight. Participants were recruited via local advertisement and mostly concerned students.

Study design: Details on study design were reported previously [16]. Briefly, in a randomized, placebo-controlled, double-blind, dose-response intervention trial, 32 healthy volunteers were allocated to a treatment with prednisolone 30 mg once daily (n=12), prednisolone 7.5 mg once daily (n=12) or placebo (n=8) for a period of two weeks using block randomization as carried out by the department of Experimental Pharmacology of the VU University Medical Center. Before and at day 14 of treatment, Angptl4 levels, glucose metabolism and total triglyceride lipolysis were measured in the fasted state and during a hyperinsulinemic-euglycemic clamp using stable isotopes. The study protocol was approved by a local ethics committee and was performed in accordance to the principles described in the declaration of Helsinki. All participating subjects gave their written informed consent prior to screening.

Experimental protocol: Design of the hyperinsulinemic-euglycemic clamp with stable isotopes is described in detail elsewhere [16]. In short, following an overnight fast and following measurement of background enrichments, a primed, continuous infusion of isotopes was started: [6,6-$^2H_2$]glucose (prime: 11 mmol/kg; continuous: 0.11 mmol/kg.min) and [1,1,2,3,3,-$^2H_5$]glycerol (prime: 1.6 mmol/kg; continuous: 0.11 mmol/kg.min) and continued until the end of the clamp. After a 2-h equilibrium period, blood samples were drawn for determination of basal glucose concentrations, isotope enrichments, glucoregulatory hormones, NEFA and Angptl4. Thereafter, a 2-step hyperinsulinemic-euglycemic clamp was started: step 1 included an infusion of insulin at a rate of 20 mU/m^2.min for 2 hours to assess hepatic insulin sensitivity; during step 2, insulin was infused at a rate of 60 mU/m^2.min for

2 hours to assess peripheral insulin sensitivity. Plasma glucose was kept at 5 mmol/l by a variable infusion of glucose 20% solution enriched with [6,6-$^{2}H_{2}$]glucose. After 2 hours of each insulin infusion, blood samples were drawn for the measurement of glucose concentrations, isotopic enrichments, glucoregulatory hormones, NEFA and Angptl4 (Figure 1).

Figure 1. Design of the two-step hyperinsulinemic clamp using stable isotopes.



Laboratory Analyses: Angptl4 was measured by ELISA as described previously [3]. Briefly, 96-well plates were coated with anti-human Angptl4 polyclonal goat IgG antibody (AF3485, R&D Systems) and incubated overnight at 4°C. Plates were washed extensively between each step. After blocking, 100 µl of 20-fold diluted human plasma was applied, followed by 2 h incubation at room temperature. A standard curve was prepared using recombinant human Angptl4 (3485-AN, R&D Systems) at 0.3 to 2.1 ng/well. Next, 100 µl of diluted biotinylated anti-human Angptl4 polyclonal goat IgG antibody (BAF3485, R&D Systems) was added for 2 h, followed by addition of streptavidin-conjugated horseradish peroxidase for 20 min, and tetramethyl benzidine substrate reagent for 6 min. The reaction was stopped by addition of 50 µl of 10% H2SO4, and the absorbance was measured at 450 nm. The intra-assay coefficient of variation (CV) for the assay was determined at 7%. Plasma glucose concentrations were measured with the glucose oxidase method using a Biosen C-line plus glucose analyzer (EKF Diagnostics, Barleben/Magedeburg, Germany). Insulin was determined on an Immulite 2000 system (Diagnostic Products Corporation, Los Angeles, CA) with a chemiluminescent immunometric assay. Plasma NEFA concentrations were measured with an enzymatic colorimetric method (NEFA-C test kit; Wako Chemicals GmbH, Neuss, Germany). [6,6-$^{2}H_{2}$] Glucose and [1,1,2,3,3,-$^{2}H_{5}$]glycerol enrichment were measured with gas chromatography-mass spectrometry (GC-MS) as described in detail elsewhere [17, 18].

Calculations: Endogenous glucose production (EGP) and the peripheral uptake of glucose (Rate of disappearance, Rd) were calculated using modified versions of the Steele equations for the non-steady state [19, 20]. Lipolysis (glycerol turnover) was computed using formulas for steady state kinetics adapted for stable isotopes [17].

Statistical analyses: Data are presented as mean values ± standard deviation (SD), or as median (interquartile range) in case of skewed distribution. To assess the effects of prednisolone treatment on various parameters, absolute changes from baseline (on treatment value minus pre treatment value) were computed for each treatment arm. Significance was tested using the Kruskal-Wallis test. Non-parametric analysis was chosen due to the relatively small number of participants and the uneven group sizes (P1 value in Table 2). Only in case of a significant finding, prednisolone 7.5 mg and prednisolone 30 mg were compared against placebo by posthoc testing, using the Mann-Whitney U test (P2 and P3 respectively in Table 2). To correct for multiple testing, Bonferroni correction was applied. The effects of insulin infusion on plasma Angptl4 levels prior to PRED treatment was tested with the paired t-test. Correlations between Angptl4 levels and parameters of glucose and lipid metabolism were calculated by regression analysis adjusting for age, BMI [21] and insulin concentrations.

Since the measurement of Angptl4 was not a primary endpoint of the original study, no formal power calculation was performed for this parameter. However, our sample size determined for the primary endpoints of the original protocol would provide a power of 82% with an estimated PRED-induced increase of 3 ng/mL in plasma Angptl4 plasma concentrations, a standard deviation of 30% and alpha set at 0.05, which we considered sufficient. The estimated changes were based on other interventions that altered Angptl4 concentrations, including fenofibrate treatment and caloric restriction [3]. All statistical analyses were run on SPSS 18.0 for Mac OS X (SPSS, Chicago, IL, USA). All statistical tests were conducted at a significance level of 0.05 (two-sided).

In vitro study

Cell culture: HepG2 cells were cultured in DMEM containing 10% fetal calf serum, 100U/mL penicillin and 100 µg/mL streptomycin. Cells were plated in 12-well plates at density of 250000 cells per well. Medium was switched to DMEM without serum one hour prior to hormone incubations. Cells were treated with dexamethasone (Sigma, Zwijndrecht, NL) and/or insulin at the indicated concentrations for 24 or 48 hours. Both Angptl4 mRNA expression and protein levels in the medium were measured. Differences were evaluated using Student's t-test.

RESULTS

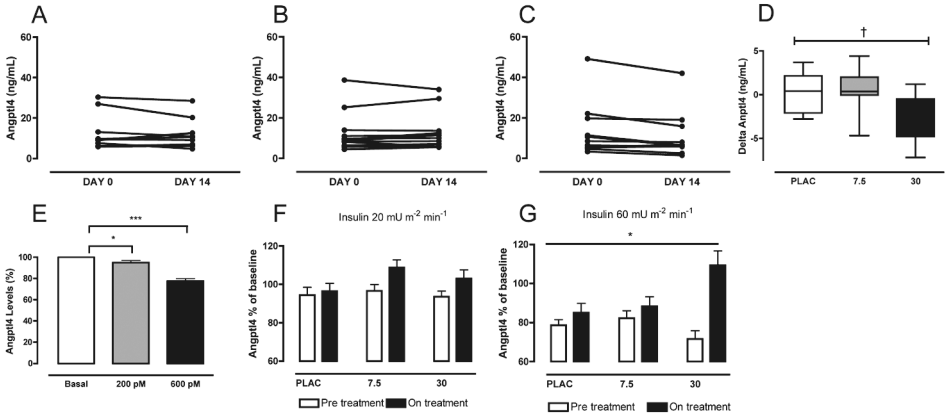
Baseline characteristics and anthropometrics: Baseline characteristics of the participants are presented in Table 1. No significant differences were observed between the groups at baseline. BMI was not changed by either treatment regimen (Table 2) [16].

Table 1. Subject characteristics at inclusion.

	Placebo	Prednisolone		
		7.5 mg	30 mg	P
N	8	12	12	
Age *(y)*	22±3	22±2	22±3	0.740
Weight *(kg)*	76.9±8.7	74.8±7.2	77.0±5.4	0.250
Height *(cm)*	184±5.6	183±7.6	186±4	0.641
BMI *(kg m^{-2})*	22.7±2.1	22.2±1.9	22.4±1.2	0.906
Lean body mass *(%)*	78.6±7.9	82.1±3.9	80.7±2.5	0.731
Fat mass *(%)*	16.9±4.7	15.9±2.8	14.8±3.5	0.531
Systolic blood pressure *(mmHg)*	118±11	125±9	127±9	0.172
Diastolic blood pressure *(mmHg)*	74±14	72±14	79±13	0.392
Fasting plasma glucose *(mmol/l)*	5.1±0.3	5.0±0.2	5.0±0.4	0.899
2-hr glucose OGTT *(mmol/l)*	4.2±0.8	4.7±0.9	4.2±1.1	0.407

Mean±SD are provided. Abbreviations: BMI: body mass index; 2-hr glucose OGTT: plasma glucose concentrations 2 h after ingestion of 75 g glucose during an oral glucose tolerance test.

Figure 2. Plasma Angptl4 levels before and at 2 weeks treatment with study medication. Angptl4 levels in the fasted state are depicted for placebo (A), prednisolone 7.5 mg daily (B) and prednisolone 30 mg daily (C). Median changes from baseline are provided in D (Box-and-Whisker plots; min-max). Prior to treatment, insulin infusion dose-dependently decreased Angptl4 levels (E). Fasting values were set at 100% (white bar), Angptl4 levels during step 1 of the clamp (200 pmol/l) are depicted in the grey bar as % of baseline, and Angptl4 levels during step 2 of the clamp (600 pmol/l) are depicted in the black bar as % of baseline. The effects of prednisolone treatment on Angptl4 concentrations during insulin infusion are shown in panel F and G. Panel F shows Angptl4 concentrations during low-dose insulin infusion, expressed as percentage change from fasting concentrations. White bar represents pre-treatment values and black bar post-treatment values. Fasting values were set at 100%. Panel G shows Angptl4 concentrations during high-dose insulin infusion, expressed as percentage change from fasting concentrations. White bar represents pre-treatment values and black bar post-treatment values. Fasting values were set at 100%. Median and SD are provided in panel (E-G). †p=0.073,*p<0.05, ***p<0.001.



Angptl4 levels: Prior to treatment, median Angptl4 levels were 10.8 (6.4-14.0) ng/mL. As observed previously [3], Angptl4 levels showed considerable inter-individual variability (Figure 2A-C). At baseline, plasma Angptl4 levels were not related to fasting glucose, insulin, NEFA and triglyceride levels (data not shown). High-dose prednisolone treatment tended to reduce plasma Angptl4 levels in the fasted state (Figure 2A-D) (P=0.073). Insulin infusion dose-dependently decreased plasma Angptl4 levels to 94% and 78% of fasting concentrations at insulin levels of 171 (153-183) pmol/l and 534 (485-564) pmol/l, respectively (Figure 2E). During low-dose insulin infusion (step 1 of the clamp), Angptl4 levels, expressed as % of fasting values, were not altered by either dosages of prednisolone (Figure 2F). However, during high-dose insulin infusion (step 2 of the clamp), insulin-mediated suppression of Angptl4 levels was blunted by prednisolone treatment (P=0.032) (Figure 2G). This effect seemed driven by the prednisolone 30 mg arm, but did not reach statistical significance in posthoc testing (data not shown). In supplemental Table 1, the absolute values of plasma Angptl4 levels during insulin infusion are depicted.

Prednisolone effects on glucose and lipid metabolism: As published previously [16], prednisolone treatment dose-dependently impaired glucose and lipid metabolism primarily by interfering with the metabolic actions of insulin. As such, insulin-stimulated suppression of EGP and lipolysis and insulin-stimulated glucose disposal were decreased in a dose-dependent manner. In addition, prednisolone 30 mg, but not prednisolone 7.5 mg, increased fasting glucose, insulin and triglyceride levels (Table 2).

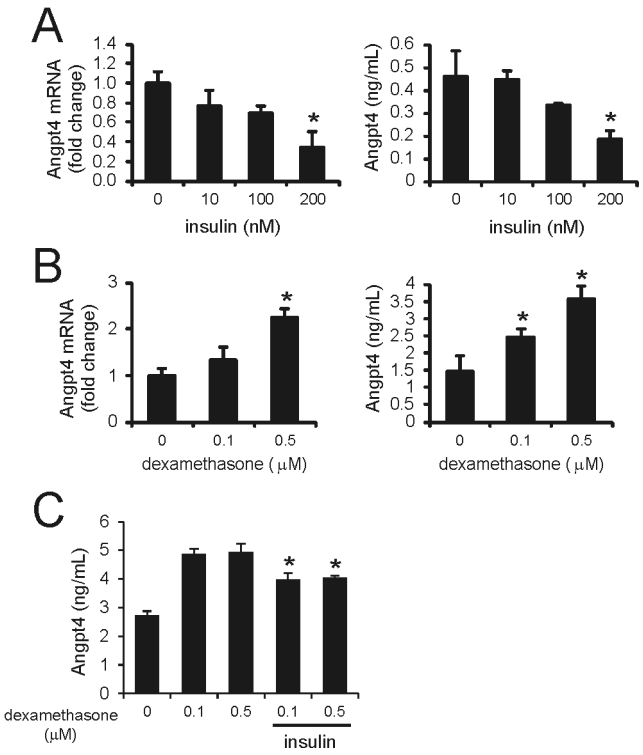
Associations between prednisolone-induced metabolic changes and Angptl4 levels: Since NEFA are potent activators of Angptl4 expression and plasma NEFAs were shown to positively correlate with plasma Angptl4 levels in large cohort studies [22], the reduction in plasma Angptl4 levels by prednisolone and insulin may be a consequence of changes in plasma NEFA levels, which were lowered by prednisolone and insulin at baseline and during the clamp, respectively. Indeed, prednisolone-induced changes in fasting NEFA levels correlated positively with prednisolone-induced alterations in fasting Angptl4 levels (β=0.444; P=0.011; R^2=0.329). Changes in plasma NEFA levels by insulin infusion, however, were not related to alterations in Angptl4 levels. Prednisolone-induced changes in plasma Angptl4 levels in the fasted state were not related to changes in fasting glucose, insulin or triglyceride levels (data not shown). Furthermore, prednisolone-induced changes in Angptl4 levels during low-and high-dose insulin infusion were not associated with changes in whole-body lipolysis and Rd (data not shown).

Table 2. Parameters of glucose metabolism and lipolysis before and during treatment with prednisolone 30 mg once daily, prednisolone 7.5 mg once daily or placebo for a period of two weeks.

	Placebo		Prednisolone 7.5 mg		Prednisolone 30 mg		P1	P2	P3
	Baseline	On-treatment	Baseline	On-treatment	Baseline	On-treatment			
BMI (kg m^{-2})	23.0±2.3	22.8±2.2	22.3±2.0	22.3±1.8	22.6±1.1	22.6±1.1	0.449	NA	NA
FPG (mmol/l)	5.0±0.2	4.8±0.3	4.9±0.2	5.1±0.3	4.7±0.2	5.4±0.3	< 0.0001	0.06	< 0.001
FPI (pmol/l)	30 (16-51)	28 (<15-38)	29 (<15-40)	19 (<15-42)	19 (<15-27)	34 (23-51)	0.0004	1.0	0.002
Basal EGP (mmol kg^{-1} min^{-1})	10.6±1.1	10.2±1.0	10.7±1.4	10.4±1.3	10.8±1.3	12.3±1.1	0.005	1.0	0.008
EGP clamp (mmol kg^{-1} min^{-1})	1.6 (0.9-3.1)	1.7 (0.7-3.1)	1.9 (0.1-3.0)	3.6 (2.2-4.4)	1.4 (0.3-3.4)	7.5 (5.8-9.9)	< 0.0001	0.014	< 0.0001
Rd (mmol kg^{-1} min^{-1})	63±9	61±11	65±9	58±10	69±12	43±9	< 0.0001	0.18	0.001
Basal lipolysis (mmol kg^{-1} min^{-1})	1.6 (1.1-2.5)	2.0 (1.9-2.3)	1.7 (1.2-2.2)	1.6 (1.5-2.1)	2.2 (1.9-3.0)	1.5 (1.4-2.0)	0.062	NA	NA
Lipolysis clamp 1 (mmol $kg^{-1}min^{-1}$)	0.6 (0.4-0.7)	0.5 (0.4-0.7)	0.4 (0.3-0.6)	0.7 (0.5-0.9)	0.5 (0.4-0.7)	0.9 (0.8-1.0)	0.005	0.09	0.004
Lipolysis clamp 2 (mmol kg^{-1} min^{-1})	0.6 (0.4-0.6)	0.4 (0.3-0.5)	0.4 (0.3-0.5)	0.5 (0.3-0.6)	0.5 (0.4-0.6)	0.7 (0.6-0.8)	0.002	0.05	0.006
NEFA basal (nmol/l)	0.5±0.2	0.5±0.1	0.4±0.1	0.5±0.1	0.6±0.2	0.4±0.1	0.017	1.0	0.11
NEFA clamp 1 (nmol/l)	0.03 (0.02-0.04)	0.03 (0.02-0.04)	0.03 (0.02-0.05)	0.05 (0.04-0.1)	0.02 (0.02-0.03)	0.12 (0.09-0.16)	< 0.0001	0.034	0.0002
NEFA clamp 2 (nmol/l)	<0.02	<0.02	<0.02	<0.02	<0.02	0.03 (0.02-0.04)	0.001	0.578	0.02
Triglyceride (mmol/l)	0.5 (0.4-0.9)	0.5 (0.3-0.7)	0.8 (0.6-1.0)	0.7 (0.6-0.9)	0.6 (0.4-0.7)	0.7 (0.6-1.2)	0.019	0.836	0.028

Mean±SD or median (interquartile range) are provided. Between-group changes from baseline were tested by Kruskal-Wallis (P1). In case of a significant finding, posthoc testing (by Mann-Whitney U) was done (P2 and P3), with P2 representing treatment-induced changes in the prednisolone 7.5 mg group compared to placebo and P3 treatment-induced changes in the prednisolone 30 mg group as compared to placebo. For plasma insulin levels, the limit of quantification (LOQ) was 15 pmol/l. In the statistical calculations, a value of 7.5 pmol/l was chosen for values below the LOQ. For plasma NEFA levels, LOQ was 0.02 nmol/l. In the statistical calculations, a value of 0.01 nmol/l was chosen for values below the LOQ. Clamp 1 denotes hyperinsulinemic euglycemic clamp with insulin infusion at 20 mU mU/m^2.min . Clamp 2 denotes hyperinsulinemic-euglycemic clamp with insulin infusion at 60 mU/m^2.min. Abbreviations: EGP: endogenous glucose production; FPG: fasting plasma glucose; FPI: fasting plasma insulin; NEFA: nonesterified fatty acids; Rd: rate of glucose disappearance.

Figure 3. Insulin and dexamethasone differentially regulate Angptl4 mRNA and secretion in hepatocytes *in vitro*. Human HepG2 hepatoma cells were treated with indicated concentrations of insulin (A) or dexamethasone (B). Relative changes in Angptl4 mRNA expression (left panel) and the absolute concentration of Angptl4 in the culture medium (right panel) were measured. C) Human HepG2 hepatoma cells were treated with indicated concentrations of dexamethasone in the presence or absence of insulin. Means and SD are provided. *p<0.05.



Effect of glucocorticoids and insulin on Angptl4 expression *in vitro*: To further investigate the isolated effects of glucocorticoids and insulin on Angtl4 expression, we studied the effect of insulin and dexamethasone in HepG2 cells (Figure 3A). Insulin dose-dependently decreased Angptl4 expression and secretion in HepG2 cells. In contrast, dexamethasone dose-dependently increased Angptl4 expression and secretion (Figure 3B). The stimulatory effect of dexamethasone on Angptl4 secretion was reduced in the presence of insulin (Figure 3C).

DISCUSSION

Little is known about regulation of Angptl4 levels except that Angptl4 production is strongly induced by fatty acids and hypoxia. In the present study we show for the first time that Angptl4

is under control of GCs and insulin in healthy humans. Accordingly, it can be hypothesized that specific metabolic actions of GCs and insulin may be partially accounted for via regulation of Angptl4.

Recently, it was shown that the Angptl4 gene contains a GRE in its 3'-untranslated region, and is a direct transcriptional target of GCs in cultured hepatocytes and adipocytes, as well as in mouse liver and white adipose tissue [15]. Upregulation of Angptl4 mRNA by GCs seemingly conflicts with data showing that cortisol markedly stimulates LPL activity in human adipose tissue *in vitro* [23]. Interestingly, Angptl4$^{-/-}$ mice were more resistant to dexamethasone-induced hypertriglyceridemia compared to their wild type littermates, leading the authors to suggest a role for Angptl4 in the metabolic effects of dexamethasone treatment [15]. Using human HepG2 cells, we confirmed that dexamethasone increases Angptl4 mRNA expression as well as protein secretion.

Surprisingly, in the *in vivo* part of this study, rather than raising fasting Angptl4 levels, high-dose prednisolone showed a tendency towards lowering Angptl4 levels. It is unlikely that this reduction is consequential to a direct effect of prednisolone treatment. High-dose prednisolone treatment induced several other metabolic effects that could have modulated fasting Angptl4 concentrations. Importantly, prednisolone 30 mg daily induced insulin resistance, fasting hyperinsulinemia and decreased fasting NEFA concentrations. Since plasma NEFA concentrations augment Angptl4 concentrations both *in vitro* and *in vivo* [1-3], decreased fasting NEFA concentrations could have therefore led to a net reduction of plasma Angptl4 concentrations. In support of this notion, in regression analysis, prednisolone-induced changes in NEFA independently predicted concomitant changes in Angptl4, whereas prednisolone treatment was not a significant predictor in this model. We show that insulin decreases Angptl4 concentrations and the observed increase in fasting insulin could also have contributed to the net reduction in fasting Angptl4 concentrations following glucocorticoid treatment. In addition, other, yet unknown, regulators that were not measured in the present study could have modulated fasting Angptl4 concentrations upon treatment with prednisolone. It should be emphasized that many aspects of the regulation of Angptl4 concentrations in humans are presently unclear.

Furthermore, we did not find a significant relationship between prednisolone-induced changes in Angptl4 levels and changes in adipose tissue lipolysis and triglyceride levels, both in the fasting state and during insulin infusion, suggesting that Angptl4 may not play a role in the metabolic effects of glucocorticoid treatment.

In addition to regulation by NEFA and GCs, Angptl4 is governed by insulin. In our study, insulin infusion dose-dependently reduced plasma Angptl4 levels. Since insulin infusion also decreased NEFA levels, it is impossible to conclude whether the decrease in Angptl4 is due to a direct effect of insulin on Angptl4 levels and/or an indirect effect through reduction of circulating NEFA. Again using HepG2 cells, it was shown that insulin dose-dependently reduced expression and secretion of Angptl4, pointing to a direct effect of insulin. Previously, insulin was already shown to inhibit Angptl4 expression in 3T3-L1 adipocytes [14]. The mechanism by which insulin regulates Angptl4 remains presently unclear.

Suppression of Angptl4 production in adipocytes and hepatocytes, which likely represent the primary sites of Angptl4 production in humans [13], may in part explain the stimulatory effect of insulin on adipose LPL and on postprandial clearance of triglyceride-rich lipoproteins [24]. Conversely, during fasting, the lack of insulin-mediated suppression of Angptl4 production may explain the decreased LPL activity [25]. Studies are underway using Angptl4-/- mice to verify the role of Angptl4 in regulation of LPL activity in the feeding-fasting cycle.

The attenuating effect of high-dose prednisolone on the insulin-mediated decrease in Angptl4 levels suggests an interaction between insulin and GCs in regulation of Angptl4 *in vivo*. Similarly, in HepG2 cells, dexamethasone prevented the reduction in Angptl4 secretion by insulin. Considering the low NEFA levels during insulin infusion and given the fact that the interaction was also observed *in vitro*, the attenuating effect of GCs is likely independent of changes in NEFA levels. Conceivably, GCs may interfere with insulin signaling and thereby reduce the effects of insulin. However, we did not observe an association between changes in Angptl4 levels and changes in insulin sensitivity, including EGP and Rd, suggesting independence of changes in insulin signaling.

We conclude that in addition to regulation by NEFAs, plasma Angptl4 is regulated by GCs and insulin, albeit in opposing ways. Whereas GCs enhance Angptl4 expression and secretion, both are reduced by insulin. Treatment with GCs attenuated the insulin-mediated decrease in Angptl4. Determination of the direct independent effect of these hormones on Angptl4 levels in humans is complicated by their interdependence. Finally, our data suggest that Angtpl4 may not be an important regulator in GC-mediated changes in lipid metabolism in humans but may contribute to insulin-mediated changes in lipid metabolism.

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Supplemental Table 1. Effect of study medication on absolute plasma Angptl4 levels in the fasted state and during insulin infusion.

	Placebo		prednisolone 7.5 mg		prednisolone 30 mg	
	Baseline	On-treatment	Baseline	On-treatment	Baseline	On-treatment
Angptl4 fasting (ng/mL)	9.5 (8.0-23.6)	11.8 (8.0-18.5)	9.0 (6.9-13.2)	10.8 (6.3-13.4)	8.6 (5.4-19.8)	6.6 (5.9-16.0)
Angptl4 clamp 1 (ng/mL)	9.1 (7.5-19.2)	10.3 (8.2-17.1)	8.7 (7.0-12.6)	12.0 (7.9-13.5)	9.3 (4.9-17.9)	6.6 (5.1-17.5)
Angptl4 clamp 2 (ng/mL)	7.8 (5.9-16.9)	8.7 (7.2-16.1)	7.3 (5.8-11.1)	9.2 (7.1-11.6)	5.9 (3.8-14.2)	7.4 (6.2-18.5)